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Authenticating Cell Lines in Ophthalmic Research Laboratories

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Authentication of cell lines in biomedical research has been elevated to a very high priority. From a review of the literature, Lacroix[1] reviewed the issue of cross-contamination of cell lines including the well known contamination of cell lines with HeLa cells,[2] and the mis-identification of the ECV304 cell line as "immortalized endothelial cells" when these cells in fact originated from T24 bladder carcinoma cells.[3] Lacroix [1] estimated that between 18 and 36% of cell lines have been misclassified. One survey at a large research institution suggested that fewer than 50% of researchers authenticate their cell lines.[4] Nardone[5] proposed recently that identification of cell lines be required of investigators before grants are awarded, and the National Institutes of Health subsequently called for researchers to authenticate cell lines as a prerequisite for grant funding.[6]

Cell lines are widely used in ophthalmic investigations. The authors' own research efforts have been focused on the biology of metastasis of uveal melanoma, and in the field of ocular oncology research, the use of cell lines is critical for at least two reasons. First, there are no animal models of spontaneous uveal melanoma that faithfully replicate the behavior of the human disease.[7] Transgenic models of uveal melanoma do not reproduce histogenesis of human uveal melanoma, and spontaneous uveal melanomas develop too sporadically in nature to be valuable in research,[8-13] or the animal melanoma tumors vary significantly from their human counterparts.[14] Therefore, uveal melanoma researchers have increasingly relied upon the implantation of animal and human melanoma cell lines into animals to model the behavior of human uveal melanoma to improve imaging techniques[15-17] and to investigate immunological[18, 19] and molecular mechanisms of tumor behavior.[20, 21]

Second, mindful of the reality that statistical associations between histological characteristics and outcome do not always indicate causality, the availability of cell lines allows ophthalmic researchers to advance from correlative studies to mechanistic investigations using advanced molecular methodologies. For example, All-Ericsson[22] identified the association between expression of insulin-like growth factor-1 by immunohistochemistry in tissue sections of uveal melanoma and adverse outcome and also demonstrated decreased tumor cell viability in uveal melanoma cell lines after interfering with the expression of ILGRF-1, pointing to the molecular significance of this histological finding. Later, Girnita et al[21] from the same group expanded upon this finding to suggest a novel therapeutic approach to uveal melanoma using the pharmacological manipulation of ILGRF-1 in human uveal melanoma cell lines.

It is possible that the behavior of long-term cell lines is not representative of the spectrum of phenotypic behaviors of neoplasms because those lines that are established *in vitro* have been

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selected for this artificial growth environment. Additionally, the phenotypic behaviors of cancer cell lines can change over time. And yet, despite these limitations, there are few satisfactory alternatives to the use of long term cell lines in modeling uveal melanoma behavior in animals or testing molecular approaches to the modifying of tumor cell behavior. Some investigators have preferred to work with short-term primary cultures to circumvent these limitations of long-term cell lines, but short-term cultures cannot be reliably shared between investigative groups, making it difficult to obtain independent validation of results on common biological substrates. Therefore, investigators have developed mechanisms for sharing long-term cell lines between groups.

In many areas of biomedical research, investigators obtain long-term cell lines from centralized repositories. However, many cell lines developed for ophthalmic research are not available from these agencies. For example, uveal melanoma cell lines are not identified in the inventories of the American Tissue Culture Collection (ATCC; www.atcc.org), the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; www.dsmz.de), the European Collection of Cell Cultures (ECACC; www.ecacc.org.uk), or the Japanese Collection of Research Bioresources (JCRB; http://cellbank.nibio.go.jp/). The European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) web site (http://www.ebi.ac.uk/ipd/estdab/directory.html) lists only four uveal melanoma cell lines (OCM3, OCM8, 92.1, and Mel-202). To complicate matters further, it is relatively difficult to establish new long-term uveal melanoma cell lines: one investigator noted that more than 60% of attempts to establish such lines were unsuccessful.[23] Therefore, as in the case of many ophthalmic research endeavors, ophthalmic oncology researchers have shared uveal melanoma cell lines with each other.

The relatively few uveal melanoma cell lines available for research have been widely circulated, but it is clear from scanning the methods sections of publications in this field that the source of the cell line does not always represent the investigator who actually developed the cell line, indicating the existence of secondary and even tertiary distributions of these scarce but vital resources. The underlying assumption behind this collegial sharing of resources is simple: a named cell line used in the United States is identical to the cell line being used in Europe or Asia with the same name. One also assumes that human lines are indeed human. We are aware of practice of propagating uveal melanoma cell lines in immunosuppressed mice, introducing the risk that murine cells may contaminate cultures or that murine pathogens can infect cultures and, at least theoretically, alter the phenotypic behavior the tumor cells. Additionally, several of the authors (MJJ, KVN, AJM, RF) have discovered that cell lines in their laboratories that were presumed to be human were in fact murine. In each case, rigorous quality control procedures in the laboratories identified the lines as non-human and no data were ever published using murine cell lines presumed to be human by any of the authors. Nevertheless, these experiences highlight the need to maintain vigilance in authenticating presumed human cell lines as indeed human.

In our own research, we have used a number of long-term uveal melanoma cell lines that have been distributed widely by the laboratories that first developed them. The OCM1, OCM3, OCM8, C918, M619, MUM2B and MUM2C cell lines were obtained by our laboratory between 2000 and 2007 as gifts from the laboratories in which these cell lines originated. We recently became aware of the mis-identification of some of these lines. Our laboratory was interested in identifying novel BRAF mutations in uveal melanoma cell lines. It has already been shown that the OCM1 cell line was positive for the V599E mutation,[24, 25] a finding of interest because in contrast with cutaneous melanoma[26] and conjunctival melanomas,[27, 28] the V599E (also known as V600E) mutation is not commonly found in uveal melanomas except for those tumors originating in the iris.[29] Recently, Maat et al[30] identified the V600E mutation in OCM1 and OCM3, and we confirmed the V599E mutation in the OCM1 and OCM3 cell

lines, and determined that the M619, C918, and MUM2B cell lines were negative for this mutation. We were surprised to discover that the MUM2C cell line was also positive for the V599E mutation, because according to the literature, the MUM2B and MUM2C cell lines were cloned from cells originating in a single hepatic metastasis.[31] This led us to question whether MUM2B and MUM2C were truly syngeneic.

The Mis-Identification Of Uveal Melanoma Cell Lines

The OCM cell lines have been described extensively in the literature as originating from primary uveal melanomas.[32] The C918 and M619 cell lines were also developed from primary uveal melanomas.[33] The MUM cell lines were reported to have originated from a hepatic metastasis from a uveal melanoma.[31] Each cell line was characterized by immunohistochemistry (S100 protein, HMB45, and Melan-A) and the ability to generate vasculogenic mimicry patterns in three-dimensional cultures.[31]

In performing internal quality controls on our lines, we validated the absence of human pathogens–Mycoplasma species, Hepatitis B Virus (HBV), Hepatitis C Virus (HCV) and Human Immunodeficiency Virus (HIV) I & II by PCR based tests performed at Charles River Diagnostics (Mycoplasma), Clongen Laboratories, Germantown, MD (HBV, HCV, HIV) and at the University of Illinois at Chicago, Department of Pathology, Molecular Genetics Laboratory (HBV, HCV, HIV). All cell lines were also found negative for thirteen murine viral pathogens including Mouse Parvoviruses MPV-1 and MPV-2, Minute Virus of Mice, Mouse Hepatitis Virus, Reovirus types 1 and 3, Lymphocytic Choriomeningitis Virus, Lactate Dehydrogenase-elevating Virus, Mouse Rotavirus, Theiler's Murine Encephalomyelitis Virus, Mousepox, Hantavirus Hantaan and Hantavirus Seoul (Mouse Essential Panel, Fluorogenic PCR Infectious Agent Assays, Charles River Diagnostics, Wilmington, MA).

Karyotypes were analyzed by growing cells under standard conditions in RPMI1640, followed by harvesting and G banding according to standard methods. Eight to 22 cells were analyzed for each cell line and karyotypes were described according to the ISCN 2005 nomenclature. Genomic DNA was extracted from each cell line using the Puregene cell and tissue kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. DNA purity and concentration were measured spectrophotometrically. Genetic identity was determined by short tandem repeat (STR) analysis using Profiler Plus and Cofiler kits (Applied Biosystems, CA) according to the manufacturer's protocol. Amplicons were generated by multiplex PCR and electrophoresed on an ABI3100 genetic analyzer. The data was analyzed using GeneMapper 4.0 software (ABI).

The OCM cell lines are no longer available from the originator[32] of these cells. For the authentication of all other cell lines, we obtained original stocks of C918 and M619 dated 1996, and compared the STR profiles of these cells with those provided to us between 2000 and 2007. We also compared STR profiles of the MUM cells provided to us by the originating laboratory with original stocks. For each of these cell lines, we detected no differences between the STR profiles of original stocks, cells provided to us by the donor laboratory, and cells currently in use in our laboratory.

The STR profiles for the cell lines tested, summarized in Table 1, indicate that OCM1 and MUM2C are from the same patient, and that M619, C918, and MUM2B are from the same patient. Historically, the MUM2B cell line has been described as originating from the liver of a patient with metastatic uveal melanoma who eventually died of his disease. We therefore obtained archival tissue from this patient's biopsy sample on file in the laboratory that provided the cells to us, extracted genomic DNA from the tissues as previously described, and compared STR profiles between this patient who died of metastatic uveal melanoma and MUM2B cells. These analyses indicate that cells designated as MUM2B are not genetically related to the patient who died of metastatic uveal melanoma. The C918 and M619 cell lines were historically

derived from primary uveal melanomas. We therefore conclude that the MUM2B cell line – from the same patient as C918 and M619 – is derived from a primary and not a metastatic uveal melanoma. We were able to extract genomic DNA from paraffin embedded samples of the tumor from which M619 was derived, and the STR profile of this tumor did not match the common STR profiles of M619, C918, or MUM2B cells. We have not been able to obtain tissue from the patient's tumor sample from which C918 was developed, but we believe that it is reasonable to infer that MUM2B, C918, and M619 are all derived from the tumor that gave rise to C918.

In addition, OCM3 and OCM8 are from the same patient. This was originally discovered by HLA typing in 1996 by one of us (MJJ) but the relationship between these cell lines has not be published previously. One of us (MJJ) contributed the cell line to the EMBL-EBI where the lines were further characterized (http://www.ebi.ac.uk/ipd/estdab/directory.html).

There are also subtle differences in the immunohistochemical profiles among even cell lines that are genetically identical according to STR analyses (Table 1). We also noticed changes in the immunohistochemical expression of melanoma markers over time. For example, the intensity of staining for S100 protein is more intense in the MUM2B cells obtained from the donor laboratory than the same cells after at least 10 passages in our own laboratory while the STR profiles of the MUM2B cells before and after passaging in our laboratory remained constant, indicating a phenotypic drift. Variations in karyotypic analyses were also detected between cell lines with identical STR profiles (Table 2 compares the karyotypes of M619 and C918), and similar to our experience with immunohistochemistry, subtle changes in the karyotypic profiles were detected after passaging while STR profiles of these cell lines did not change after passaging in our laboratory.

Implications for the Uveal Melanoma Research Community

To the best of our knowledge, we are the first group to publish STR profiles of commonly used uveal melanoma cell lines, and we encourage others in this field to publish STR analyses of the other uveal melanoma cell lines used in research. Because the cell lines that we describe in this communication have been distributed throughout the world by various laboratories, we encourage investigators who work with these cells to perform STR analyses on their lines to authenticate the identity of their materials. STR analyses are relatively inexpensive and easier to perform than karyotyping. Karyotypic analyses do have an important role in ophthalmic oncology research, especially with regard to the status of chromosomes as prognostic markers.[34] However, the issue here is not that of a biomarker of malignant behavior, but rather a matter of identities of the biological substrates of experiments. Finally, although investigators should not refer to MUM2B cells as originating from a metastasis, it is appropriate to refer to cells labeled as C918, M619, or MUM2B as either invasive or aggressive.

With only two exceptions, the publications from our group that utilized these cell lines dealt with phenotypic behaviors.[35-43] The conclusions of these papers are therefore still valid in the context of comparing the behavior of invasive/aggressive uveal melanoma cells and non-invasive/poorly aggressive uveal melanoma cells. In one study we compared gene expression between MUM2B (C918) and MUM2C (OCM1) – valid comparisons between a highly invasive/aggressive cell line (C918) and a poorly invasive/aggressive cell line (OCM1).[36] In another study we compared gene expression between two highly invasive cell lines (M619 and MUM2B) and a poorly-invasive/aggressive cell line (OCM1), but even this comparison is valid because the comparisons were made between two phenotypic variants of the same highly-invasive/aggressive uveal melanoma cell line (C918) as defined by identical STR profiles and a poorly-invasive uveal melanoma cell line (OCM1) with a different STR profile.[38]

Implications and Recommendations for the General Vision Research Community

Although the incident we describe in this communication came to light in the domain of uveal melanoma research, the imperative to authenticate cell lines extends to all disciplines within the vision research community. It is important that vision researchers using cell lines authenticate their lines against a published standard, assure journal editors, journal readers, and granting agencies that their cell lines have been authenticated, and take measures to eliminate the risks of cell line cross-contamination in their laboratories.

The requirement for investigators to authenticate their cell lines against a standard requires a central repository of either cell lines or data by which cell lines can be authenticated. Although investigators funded by the National Institutes of Health are expected to share their cell lines with those who request these resources, it may not serve the interests of the vision research community to require that the cell lines be deposited physically with a central repository such as the ATCC. The number of ophthalmic investigators using a cell line of interest is limited compared with the numbers of investigators who utilize cell lines typically stocked in centralized repositories. Moreover, the withdrawal of cell lines from repositories is associated with a fee, thus limiting access to these cells to investigators of limited financial means.

In our investigations, we relied on STR methodology to characterize the uveal melanoma cell lines for the reasons summarized recently by Josephson et al[44]: (1) STR analysis is a simple, relatively inexpensive test available in kit form from a number of vendors; (2) STR analysis readily identifies male and female samples; (3) STR analysis permits the discrimination of fewer than 1 in 10^8 individuals;[45] and (4) STR analysis is now used as an authentication benchmark by central cell line repositories. The ATTC now provides a comprehensive STR database for cell lines and encourages investigators to fingerprint cells in their labs and to compare the analyses against the information published and available on-line (http://www.atcc.org/CulturesandProducts/CellBiology/STRProfileDatabase/tabid/174/Default.aspx). The ECACC uses a combination of methods to authenticate cell lines including isoenzyme analysis, multilocus DNA fingerprinting, and STR profiling (http://www.ecacc.org.uk/). The DSMZ provides investigators with information on cell lines in their repository including cytogenetics, DNA typing (STR profiling), and immunophenotyping, in addition to speciation and mycoplasma detection and viral analyses (http://www.dsmz.de/human_and_animal_cell_lines/main.php?contentleft_id=21).

Although STR profiling is now emerging as a standard for cell line authentication, other data is useful for investigators. The EMBL-EBI lists STR profiles along with many genomic and molecular profiles including high resolution HLA typing and HLA and related genes surface expression. The publication by de Waard-Sibienga [46] et al in 1995 is an example of a thorough description of a novel uveal melanoma cell line in which the authors provide detailed analyses of HLA profiles. Because of the considerable interest in uveal melanoma immunology, the HLA profiles of novel cell lines, if known, should be shared with the research community.

Requiring investigators to deposit STR analyses into a centralized data repository that can be accessed by the research community anonymously follows the precedent of requiring investigators to post gene expression profiles to centralized data warehouses. In general, it is not sufficient that investigators make STR profiles available on their laboratory websites for several reasons: (1) investigators may move from one organization to another, creating orphan links to critical STR profile data, (2) website themselves may not be maintained optimally resulting in outdated links that do not provide data access, and (3) it is possible for investigators to identify those individuals who seek data about STR profiles from laboratory-maintained

websites. The creation of a web-based centralized cell authentication data repository by an "honest broker" would allow for anonymous access of information of importance to the vision research community. Because the number of cell lines of interest to the vision research community is relatively limited, organizations such as ARVO may wish to consider methods by which such a data repository can be constructed and maintained. If the STRs of cell lines were available, then it would be reasonable to require that researchers periodically validate the authenticity of the lines in their laboratories.

It is also reasonable to require that authors declare in manuscripts submitted for publication that cell lines used in their studies (1) have been validated as to species (*i.e.*, the cells are human and not murine if the subject of the investigation is the behavior of human cells), (2) have been validated as pathogen-free, (3) the cells have been authenticated by STR analysis, if human, against a benchmark standard, and (4) the investigators follow standard operating procedures including the implementation of quality management programs. This declaration should be no more burdensome than requiring investigators who enroll human subjects or animals in their studies to state that their research conforms to the Declaration of Helsinki or the ARVO policy on the handling of animals.

If research laboratories followed the same set of standard operating procedures required of clinical diagnostic laboratories that use human cell lines – such as diagnostic cytogenetics laboratories where the risk of cell line cross-contamination would result in misdiagnoses of horrific proportions – then it is likely that the integrity of cell lines in research laboratories would be improved significantly.[47] Again, organizations such as ARVO can assume a leadership role in developing and publishing standard operating procedures and quality management programs for investigators who use cell lines

Dealing with the consequences of cross-contaminated cell lines is wasteful of time and dwindling research resources. The vision research community is now challenged to design and implement strategies to reduce the risk of mis-identified cell lines in ophthalmic research.

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Table 1

Genetic identity and phenotypic characterization of uveal melanoma cell lines

STR - Profiler Plus Analysis							
	OCM1	**MUM2C**	**OCM3**	**OCM8**	**MUM2B**	**M619**	**C918**
D3S1358	14, 16	14, 16	16, 18	16, 18	16	16	16
vWA	18	18	16, 19	16, 19	16, 17	16, 17	16, 17
FGA	21	21	19	19	20, 21	20, 21	20, 21
AMEL	X	X	X, Y	X, Y	X	X	X
D8S1179	13	13	13	13	13	13	13
D21S11	30	30	28, 29	28, 29	30	30	30
D18S51	13, 18	13, 18	12, 16	12, 16	15	15	15
D5S818	11, 12	11, 12	11, 13	11, 13	11, 12	11, 12	11, 12
D13S317	12	12	11, 12	11, 12	11	11	11
D7S820	8, 10	8, 10	10	10	12	12	12
STR - COfiler Analysis							
	OCM1	**MUM2C**	**OCM3**	**OCM8**	**MUM2B**	**M619**	**C918**
D3S1358	14, 16	14, 16	16, 18	16, 18	16	16	16
D16S539	9	9	9, 12	9, 12	8, 12	8, 12	8, 12
AMEL	X	X	X, Y	X, Y	X	X	X
THO1	6, 7	6, 7	7	7	8, 9.3	8, 9.3	8, 9.3
TPOX	8, 11	8, 11	8, 12	8, 12	11	11	11
CSF1PO	11	11	10, 12	10, 12	10, 11	10, 11	10, 11
D7S820	8, 10	8, 10	10	10	12	12	12
Phenotype							
	OCM1	**MUM2C**	**OCM3**	**OCM8**	**MUM2B**	**M619**	**C918**
S-100*	+ all cells	+ all cells	NP	+ all cells	+ all cells	+ all cells	+ all cells
HMB-45	+ all cells	+ all cells	NP	+ all cells	+ variable	-	-
Melan-A Vasculogenic	+ all cells	+ all cells	NP	+ all cells	+ variable	-	-

NP = not performed. OCM1 and MUM2C originate from the same patient, OCM3 and OCM8 originate from the same patient, and MUM2B, M619 and C918 originate from the same patient.

Table 2

Representative uveal melanoma cell line karyotpic profiles

Cell Line	Comments	Karyotype
C918	Cells as received from the donor laboratory, passage 10	46,X,-?,inv(5)(p15q33), der(7;17)(p10;q10),del(10)(q22q24), der(13;?16)(q10;q10), der(18)t(9;18)(q?31;q22),+add(21)(p11.2),del(22)(q12),+del(22)(q12)[9]
C918	Cells in our laboratory, passage 10	47,X,-?,+del(3)(p13),inv(5)(p15q33), der(7;17)(p10;q10),i(8)(q10),del(10)(q22q24), der(13;?16)(q10;q10),der(18)t(9;18)(q?31;q22), +add(21)(p11.2),del(22)(q12),+del(22)(q12)[10]
M619	Cells received from contributing laboratory, passage 5	82~91<4n>,XX,-?,-?,+del(3)(p13),-4,inv(5)(p15q33)x2, der(7;17)(p10;q10)x2,i(8)(q10)x2,-9, del(10)(q22q24)x2,del(12)(q13q2?4.1)x2, der(1 3;?16)(q10;q10)x2,add(14)(p13)x2,-16, der(18)t(12;18)(q13;q22)x2,+add(21)(p11.2)x2, -22x2,+2~5mar[10]
M619	Cells in our laboratory, passage 10	80~88<4n>,X,-X,-?,-?,add(2)(q3?7),-4,inv(5)(p15q33)x2, der(7;17)(p10;q10)x2,i(8)(q10),+i(8)(q10), del(10)(q22q24)x2, der(11)t(11;15)(p13;q11.2), der(13;?16)(q10;q10)x2,-16, der(14)t(7;14)(p13;p11.2),-15, der(18)t(12;18)(q13;q22)x2,+add(21)(p11.2) x2,-22x2,+1~5mar[11]